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# [26] Eukaryotic Cloning Vectors Derived from Bovine Papillomavirus DNA

*By* PETER M. HOWLEY, NAVA SARVER, and MING-FAN LAW

SV40, the human adenoviruses, and the human herpesviruses are currently being exploited and developed as viral vector systems for the transient expression of foreign genes in permissive cells. Similarly, the LTR segments of cloned retrovirus DNAs are being used as components of viral vectors to enhance transformation efficiency and integration into the host chromosome. The finding that bovine papillomavirus (BPV) genome remains exclusively extrachromosomal as a circular plasmid in transformed mouse cells[1] suggested the potential utility of the papillomavirus DNAs as plasmid cloning vectors for introducing foreign DNA fragments into cells susceptible to papillomavirus-mediated transformation. We demonstrated the utility of the subgenomic transforming segment of the BPV genome as a eukaryotic cloning vector using the 1.62 kb fragment of the rat preproinsulin gene ($rI_1$).[2] We describe here the use of BPV as a cloning vector and recent experiments that permit the shuttling of plasmids between eukaryotic and bacterial cells.

## Materials and Reagents

*BPV DNA*. The BPV-1 genome was cloned from a cutaneous bovine fibropapilloma (isolate 307).[3] The genome consists of 7944 base pairs, and the complete nucleotide sequence is now known.[4] Only a specific 69% subgenomic segment of the genome mapping between the *Bam*HI and *Hin*dIII sites is required for transformation of mammalian cells[5] and au-

[1] M.-F. Law, D. R. Lowy, I. Dvoretzky, and P. M. Howley, *Proc. Natl. Acad. Sci. U. S. A.* **78**, 2727 (1981).
[2] N. Sarver, P. Gruss, M.-F. Law, G. Khoury, and P. M. Howley, *Mol. Cell. Biol.* **1**, 486 (1981).
[3] P. M. Howley, M.-F. Law, C. A. Heilman, L. W. Engel, M. C. Alonso, W. D. Lancaster, M. A. Israel, and D. R. Lowy, *in* "Viruses in Naturally Occurring Cancers" (M. Essex, G. Todaro, and H. zur Hausen, eds.), p. 233. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1980.
[4] E. Y. Chen, P. M. Howley, A. Levinson, and P. H. Seeburg, *Nature* (*London*) **299**, 529 (1982).
[5] D. R. Lowy, I. Dvoretzky, R. Shober, M.-F. Law, L. Engel, and P. M. Howley, *Nature* (*London*) **287**, 72 (1980).




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tonomous extrachromosomal replication.[1] A detailed restriction endonuclease map of the BPV-1 genome indicating the specific subgenomic transforming segment is shown in Fig. 1.

*Cells.* We have used the mouse C127 cells exclusively in our transformation studies owing to the distinctive characteristic of the BPV-induced

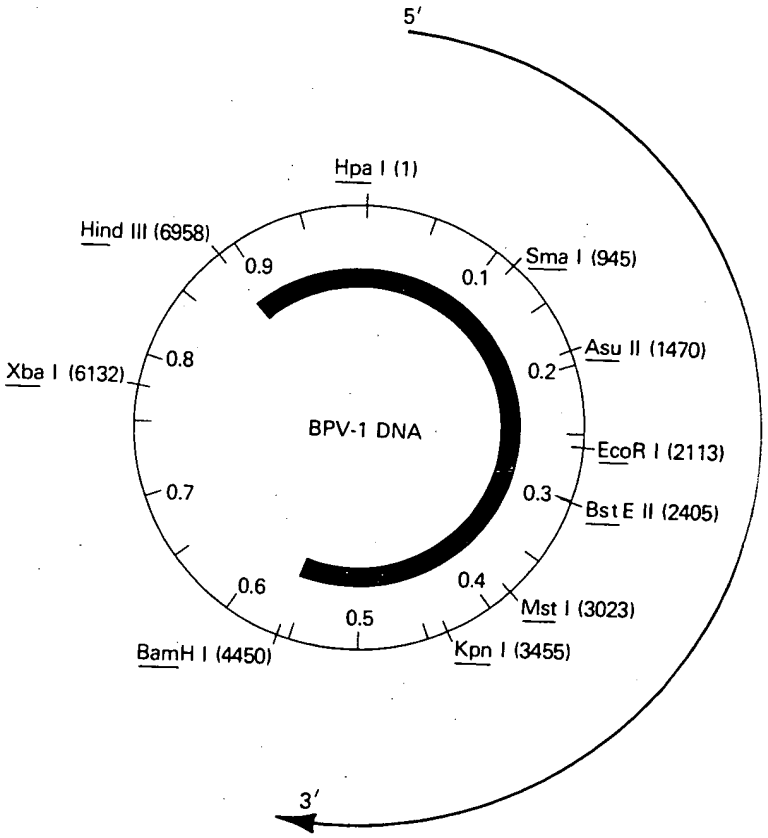



FIG. 1. Restriction endonuclease map of the BPV-1 genome. The restriction endonuclease sites present once in the genome are indicated. The map units and base numbering are derived from the sequence using the *Hpa*I site as the 0/1.0 map unit.[4] The subgenomic (*Bam*HI/*Hin*dIII) transforming segment is indicated by the filled bar.[5] The transcription direction and the extent of the genome represented by polyadenylated transcripts found in BPV-1 transformed mouse cells are indicated by the outer arrow.[8] Restriction endonucleases that do not recognize sites in the BPV-1 genome include *Bal*I, *Sst*I, *Sst*II, *Pvu*I, *Nru*I and *Xho*I.

transformed phenotype.[6] NIH-3T3 cells[6] and rat 3T3 cells[7] are also susceptible to BPV-mediated transformation.

## Methods

### *Construction of Hybrid DNAs*

The BPV DNA used as a vector in all our experiments to date has been the 69% subgenomic transforming segment of the BPV-1 genome, extending from the *Hin*dIII site to the *Bam*Hi site.[5,8] Modification of the termini of this segment with synthetic linkers does not reduce the efficiency of transformation. Earlier studies in this laboratory have indicated the pBR322 sequences covalently linked to the subgenomic transforming segment of the bovine papillomavirus is inhibitory to cellular transformation. For this reason, hybrid DNAs have been constructed in such a way as to allow for the physical separation of the pBR322 sequences from the remainder of the BPV-hybrid molecule. This is accomplished by modifying the termini between the BPV-hybrid and the pBR322 portions of the DNA to homologous restriction endonuclease recognition sites that are not present elsewhere in the molecule. Cleavage with the appropriate restriction endonuclease, therefore, separates the molecule into its pBR322 and BPV-hybrid moieties. The inhibition of transformation occurs only when the pBR322 sequences are covalently linked to the BPV-hybrid portion, thus it is not necessary to purify the viral DNA sequences away from the pBR322 sequences prior to transfection.

The steps involved in a typical construction of a hybrid DNA containing pBR322 and BPV sequences is diagrammed in Fig. 2.[2] The source of the DNA to be cloned is the 5.3 kb fragment of the rat preproinsulin gene ($rI_1$)[9] cloned in pBR322 at the *Bam*HI site. A 1.62 kilobase (kb) segment containing the coding sequences of the $rI_1$ gene, its intervening sequence, and the regulatory signals at the 5′ and 3′ ends was generated from the cloned 5.3 kb DNA by a *Bam*HI and *Hin*cII restriction endonuclease digestion. After two purification steps through agarose gels, the DNA was electroeluted from the gel, extracted with phenol, and precipitated with

[6] I. Dvoretzky, R. Shober, and D. R. Lowy, *Virology* **103**, 369 (1980).
[7] B. Binétruy, G. Rautmann, G. Menequzzi, R. Breathnach, and F. Cuzin, *in* "Eukaryotic Viral Vectors" (Y. Gluzman, ed.), p. 87. Cold Spring Habor Laboratory, Cold Spring Harbor, New York (1982).
[8] C. A. Heilman, L. Engel, D. R. Lowy, and P. M. Howley, *Virology* **119**, 22 (1982).
[9] P. Lomedico, N. Rosenthal, A. Efstratiadis, W. Gilbert, R. Kolodner, and R. Tizard, *Cell* **18**, 545 (1979).

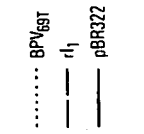
pBPV68T
BAM
HIND III
BAM/HIND III
BPV68T
p rl1 (1.62)
rl1
LIGASE
pBR322
pBPV68T – rl1
HINC II
1.62 KB
p rl1 (5.3)
BAM/HINC II
HIND III LINKERS
BPV68T
rl1
pBR322

ethanol. Synthetic *Hin*dIII linkers (Collaborative Research) were joined to the *Hin*cII site, and the products were digested with *Hin*dIII to generate ends with monomeric linkers.[10] The modified 1.62 kb fragment was then ligated to the 4.0 kb fragment of the *Bam*HI–*Hin*dIII cleaved pBR322 DNA, and the ligation mixture was used to transform *Escherichia coli* K12 (strain HB101).[11] Plasmid DNA from ampicillin-resistant, tetracycline-sensitive colonies was isolated after an amplification step with chloramphenicol[12] and analyzed with restriction enzymes for the presence of the 1.62 kb fragment insert. One such plasmid, $prI_1$ (1.62 kb) was selected for further study.

The 69% transforming region of BPV-1 was excised from $pBPV_{69T}$ by *Bam*HI and *Hin*dIII codigestion, purified on an agarose gel, and ligated to gel-purified 1.62 kb fragment of $prI_1$ (1.62 kb). After digestion with *Hin*dIII, the products were ligated to *Hin*dIII-cleaved pBR322, and a ligation mixture was used to transform susceptible *E. coli* HB101 cells. DNAs from ampicillin-resistant, tetracycline-sensitive colonies were analyzed for the presence of the 1.62 kb insert, and the positive colony thus identified ($pBPV_{69T}$-$rI_1$) was amplified in *E. coli* HB101.

A number of different $BPV_{69T}$ cloning vectors have been generated in

[10] T. Maniatis, R. C. Hardison, E. Lacy, J. Lauer, C. O'Connel, D. Quon, G. K. Sim, and A. Efstratiadis, *Cell* **15**, 687 (1978).
[11] K. W. Hutchinson and H. O. Halvorson, *Gene* **8**, 267 (1980).
[12] D. B. Clewell, *J. Bacteriol.* **110**, 667 (1972).



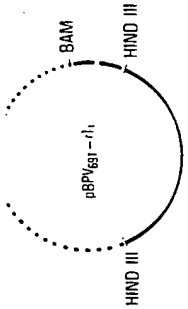





FIG. 2. Construction of $pBPV_{69T}$-$rI_1$. A 1.62-kb segment containing the coding sequences of the $rI_1$ gene, its intervening sequence, and the regulatory signals at the 5′ and 3′ termini was generated from the cloned 5.3-kb DNA by a *Bam*HI + *Hin*cII digest. After two purification steps through agarose gels the DNA was electroeluted from the gel, extracted with phenol, and precipitated with ethanol. $^{32}$P-labeled synthetic *Hin*dIII linkers (Collaborative Research) were joined to the *Hin*cII site, and the products were digested with *Hin*dIII to generate tails with monomeric linkers. Modified 1.62-kb fragments were then ligated to the 4.0-kb fragment of *Bam*HI + *Hin*dIII-cleaved pBR322, and the ligation mixture was used to transform *Escherichia coli* K12 strain HB101. Plasmid DNA from ampicillin-resistant, tetracycline-sensitive colonies was isolated after an amplification step with chloramphenicol and analyzed with restriction enzymes for the presence of the 1.62-kb fragment. A recombinant plasmid, $pBPV_{69T}$, containing the 69% transforming region of bovine papillomavirus 1 DNA, has been described.[3] Viral DNA was excised from this recombinant by *Bam*HI + *Hin*dIII digestion, purified on an agarose gel, and ligated to the gel-purified, 1.62-kb fragment of $prI_1$. After digestion with *Hin*dIII, the products were ligated to *Hin*dIII-cleaved pBR322, and the resulting DNA was used to transform *E. coli* strain HB101. DNA from ampicillin-resistant, tetracycline-sensitive colonies was isolated and analyzed with restriction endonucleases. One of the colonies containing the recombinant DNA ($pBPV_{69T}$-$rI_1$) was isolated, amplified, and used for subsequent studies. Reprinted from Sarver *et al.*,[2] with permission.

TABLE I
$BPV_{69T}$ CLONING VECTORS

| Vector[a] | Plasmid segment | BPV-1 insert | pBR322/BPV junction[b] | Clonable sites[c] |
|---|---|---|---|---|
| $pBPV_{69T}$ (52-1) | pBR322 | 69% transforming segment | *Xho*I | *Bam*HI |
| $pBPV_{69T}$ (69-6) | pBR322 | 69% transforming segment | *Sal*I | *Bam*HI<br>*Xba*I<br>*Xho*I |
| $pBPV_{69T}$ (70-20) | pBR322 | 69% transforming segment | *Sal*I | *Bam*HI<br>*Hin*dIII<br>*Xho*I |

[a] Unpublished data of M.-F. Law and P. Howley.
[b] Restriction endonuclease sites generated by *Escherichia coli* DNA polymerase I (Klenow fragment) fill-in reaction and synthetic linkers at junction between pBR322 and BPV-1 DNA sequences. Cleavage with this enzyme separates the molecule into hybrid DNA and pBR322 moieties.
[c] Unique sites present on BPV-1 side of pBR322/BPV-1 DNA junction suitable for the insertion of foreign DNA segments.

pBR322 or into pML2, a deletion derivative of pBR322.[13] These hybrid DNAs have been constructed using fill-in reactions and synthetic linkers to create new restriction endonuclease sites at the junction of the BPV and pBR322 sequences. Unique clonable restriction endonuclease sites are located in these plasmid vectors for the insertion of foreign DNA segments to generate hybrids, which can then be separated from the pBR322-derived sequences by a single restriction endonuclease cleavage.[14] A listing of some of these vectors are presented in Table I.

### *Transfection of Eukaryotic Cells*

Several methods are currently available for delivering naked DNA into eukaryotic cells. These include the calcium phosphate method,[15] the DEAE-dextran method,[16,17] and more recently the protoplast fusion technique.[18] We have tested these three methods, and they are discussed.

As mentioned above, a variety of cell types are susceptible to BPV-

[13] M. Lusky and M. Botchan, *Nature* (*London*) **293**, 79 (1981).
[14] M.-F. Law, B. Howard, N. Sarver, and P. M. Howley, *in* "Eukaryotic Viral Vectors" (Y. Gluzman, ed.), p. 79. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.
[15] F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).
[16] J. H. McCutchan and J. S. Pagano, *J. Natl. Cancer Inst.* **41**, 351 (1968).
[17] L. M. Sompayrac and K. J. Danna, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 7575 (1981).
[18] W. Schaffner, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2163 (1980).

mediated transformation. These include the C127 cells derived from a mammary tumor of an RIII mouse, the NIH-3T3 cells,[6] and the Fisher rat 3T3 cells.[7] In our laboratory, the bulk of our experience has been with the C127 cells. To ensure reproducibility from experiment to experiment, we routinely use early-passage cells that have been frozen in parallel. We carry the cells from 1 vial for approximately 8 passages before returning to an early-passage freeze-down. One day prior to transformation, the cells are split and seeded at approximately $5 \times 10^5$ cells per 60-mm tissue culture dish. This initial cell density gives a 50–75% confluency level after 24 hr. The medium is changed 3–4 hr prior to transfection.

*Calcium Precipitation.* The procedure is a modification of that first described by Graham and van der Eb.[15] A sterile 2× $CaCl_2$ solution is dispensed into a sterile tube, and carrier DNA (i.e., calf thymus DNA or salmon sperm DNA) and the desired amount of recombinant DNA are added such that the final DNA concentration in the transfection cocktail will be 20 μg/ml. A second tube contains an equal volume of sterile 2× HeBS solution [280 m*M* NaCl, 50 m*M* HEPES (Sigma), 1.5 m*M* $Na_2HPO_4$, pH 7.10 ± 0.05]. This buffer may be stored in aliquots at −70° in polyethylene tubes until use. The sterile 2 M calcium chloride solution should either be made fresh or be stored at −20 in plastic tubes until ready for use.

The coprecipitate is formed by adding the 2× $CaCl_2$–DNA solution to the tube containing the equal volume of the 2× HeBS solution. This is accomplished by inserting a 1-ml sterile cotton-plugged pipette into the mixing tube containing the 2× HeBS solution and gently agitating the mixture by blowing bubbles while the 2× DNA-calcium chloride solution is slowly added. The calcium phosphate DNA precipitate is allowed to form without further agitation for 45 min at room temperature. It is then mixed gently and applied (0.5 ml per plate), at an equivalent concentration of approximately 1μg of recombinant DNA per plate, directly into the medium (5 ml) of the 60-mm plate. The precipitate is allowed to remain on the cells for 4 hr at 37°.

The efficiency of DNA-mediated transformation with calcium precipitate is increased by treatment with DMSO or glycerol 4 hr after transfection.[19] We therefore have used this method routinely in our transfection studies. Since the toxicity levels of these agents may vary with different cell types, the toxicity should be titrated with the desired agent and a subtoxic dose applied to the cells. The shock treatment is as follows. Four hours after transfection, the medium is removed, the cells are washed once with fresh medium containing 10% fetal calf serum and drained.

[19] N. D. Stowe and N. M. Wilkie, *J. Gen. Virol.* **33**, 447 (1976).

FOCUS OF C127 CELLS
TRANSFORMED BY BPV69T - rl1
NORMAL C127 CELLS

Sterile DMSO (25% in 1 × HBS) is prepared, and a 1-ml aliquot is applied to the monolayer for 4 min at room temperature with frequent swirling of plates. It is then removed and the cells are washed with medium and refed with medium containing 10% fetal calf serum. Alternatively, the shock may be accomplished using 20–25% glycerol in DME/10% fetal calf serum overlaid on the cells for 1 min. Either technique routinely results in a 3 to 5 fold enhancement in the transformation frequency.

*DEAE-Dextran.* We have not been able successfully to transform C127 cells by introducing the cloned BPV-1 DNA using DEAE-dextran as a facilitator. We have attempted to transfect mouse cells using DNA at a high concentration (1 mg/ml) of DEAE-dextran for 15 min as originally described by McCutchan and Pagano,[16] as well as at a lower concentration (200 μg/ml) for a prolonged time (8 hr).[17] With neither protocol were BPV transformants induced.

*Protoplast fusion.* Polyethylene glycol (PEG)-mediated fusion of cells with bacterial protoplasts[18] is an alternative way of effectively delivering BPV-pBR322 hybrid DNAs to C127 cells and rat 3T3 cells.[7] The efficiency of transformation was comparable with *E. coli* containing BPV molecules cloned either into pBR322 or into a derivative pML-2.[13] Further modifications of the protoplast fusion technique that would result in higher levels of transformation would make this an extremely useful approach in the future.

### *Selection of Transformants*

Foci of transformed cells can be recognized 6–7 days after transfection and are of sufficient size to be isolated by day 14 or 16. An illustration of an early transformed focus induced by the $BPV_{69T}$-$rI_1$ hybrid DNA on C127 cells is shown in Fig. 3. Individual foci are trypsinized, and subsequent cell lines are established by single cell cloning.

### *Analysis of Gene Expression*

A typical analysis of the lines established from single cell clones involves an analysis of the state of the hybrid DNA within the transformed cell line, an analysis of the RNA transcripts derived from the foreign DNA inserted into the cells, and finally an analysis of any protein products.

FIG. 3. Morphological appearance of untransformed C127 cells (left panel) and a $BPV_{69T}$-$rI_1$-induced focus in the same cell line (right panel). The photomicrographs were taken 12 days after transfection (original magnification × 56).

*Analysis of DNA in Transformed Cell Lines*. Total cellular DNA is extracted from the cell lines by the method of Gross-Bellard[20] and analyzed by the Southern blotting technique.[21] The DNA is either treated with a restriction endonuclease or lightly sheared by passing 10 times through a 25-gauge needle and electrophoresed through a 0.6% agarose gel. After depurination and denaturation *in situ*, the DNA is transferred onto a nitrocellulose membrane (BA85, Schleicher & Schuell Co). The nitrocellulose filters are then hybridized under standard conditions with a nick-translated $^{32}$P-labeled probe prepared according to the technique of Rigby *et al.*[22] After hybridization, the filters are washed and exposed to X-ray films as previously described.[1]

*Transcriptional Analysis*. Polyadenylated cytoplasmic RNA can be isolated from the transformed cells and the RNA analyzed for the presence of transcripts complementary to the cloned foreign DNA segment using the S1 endonuclease and exonuclease VII mapping methods of Berk and Sharp.[23] An example showing the S1 endonuclease and exonuclease VII analysis of rat preproinsulin RNA in mouse cells transformed by the BPV-rI$_1$ hybrid DNA is shown in Fig. 4.[24] In this analysis, polyadenylated RNA is hybridized to a uniformly labeled $^{32}$P-labeled probe under conditions that will favor the formation of RNA–DNA duplexes. Hybrids thus formed are treated with either S1 nuclease or exonuclease VII. The former hydrolyzes the single-stranded DNA tails at the 3′ and 5′ termini of the duplex molecule as well as the unhybridized intervening sequences within the gene. The resulting products, analyzed on alkaline agarose gels, thus represent the exons present in specific messenger RNAs (mRNAs). Exonuclease VII, on the other hand, digests only the single-stranded termini, but not internal single-stranded loops. The size of the fragment, therefore, corresponds to the size of the intron plus the exons of the gene.

*Protein Analysis*. The protein analysis can be done in a variety of ways. If the inserted foreign DNA encodes a biologically active protein, a biological assay can be utilized to assess the synthesis of the protein. Alternatively, a radioimmunoassay may be utilized to detect small levels of the protein either in cellular extracts of the transformed cells or in the media. Finally, the identify of the protein can be established by specific

[20] M. Gross-Bellard, P. Oudet, and P. Chambon, *Eur. J. Biochem.* **36**, 32 (1973).
[21] E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
[22] P. Rigby, D. Rhodes, M. Dieckmann, and P. Berg, *J. Mol. Biol.* **113**, 237 (1977).
[23] A. J. Berk and P. A. Sharp, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1274 (1978).
[24] N. Sarver, P. Gruss, M.-F. Law, G. Khoury, and P. M. Howley, *in* "Developmental Biology Using Purified Genes" (D. Brown and C. R. Fox, eds.), p. 547. Academic Press, New York, 1981.

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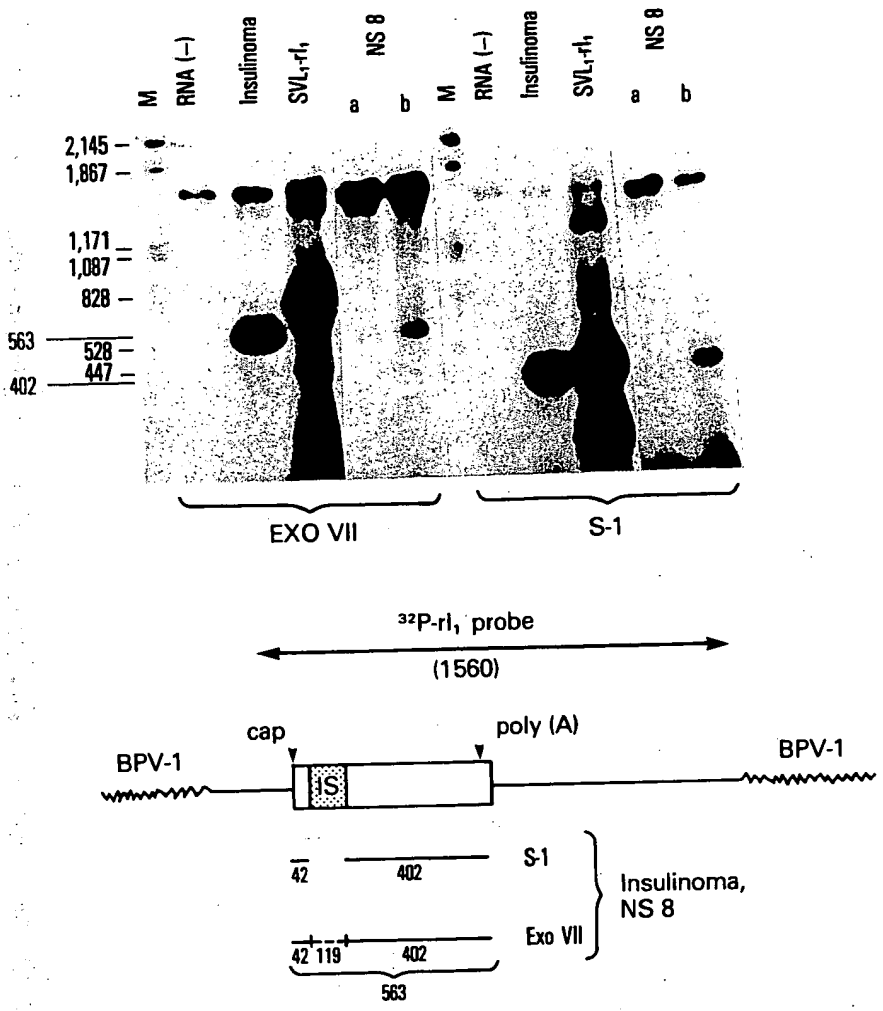


FIG. 4. Poly(A)-selected RNA from $2 \times 10^6$ and $5 \times 10^6$ cells (lanes a and b, respectively) were mixed with 10,000 cpm of $^{32}$p-labeled rat preproinsulin DNA ($2 \times 10^6$ cpm/$\mu$g) purified from recombinant SVL1-rI$_1$ virus by *Hae*II–*Bam*HI digestion.[2] The mixture was precipitated with ethanol, resuspended in 20 $\mu$l of formamide buffer, and hybridized for 3 hr at 50°. RNA–DNA duplexes were treated with endonuclease S1 or exonuclease VII,[23] and the digests were analyzed by electrophoresis through a 1.4% alkaline agarose gel. The gel was exposed for 72 hr at −70°. Numbers to the left of the gel indicate the size in base pairs of SV40 DNA segments. The 1560 base species represents the self-annealed $^{32}$P-labeled DNA probe. DNA protected by authentic preproinsulin mRNA is 402 nucleotides in the case of endonuclease S1 analysis and 563 nucleotides for exonuclease VII analysis. RNA(−): probe contained no added RNA; insulinoma: RNA from rat insulinoma cells; SVL1-rI$_1$: RNA from AGMK cells infected with SV40-insulin recombinant DNA; NS8: RNA from BPV$_{697}$-rI$_1$ transformed cells. The diagram depicts the classes of DNA fragments expected if only insulin regulatory signals are involved in transcription. Reprinted, with permission, from Sarver *et al.*[24]

radiolabeling and immunoprecipitation. The identity of the protein was established as that of rat proinsulin by competitive immunoprecipitation studies followed by analysis on SDS–polyacrylamide gels. Cells in 100-ml plates were washed for 3 hr before labeling with Earle's balanced salts containing 5% normal medium and 2% dialyzed fetal bovine serum and labeled in the same medium with 200 $\mu$Ci of L-[$^{35}$S]cysteine per milliliter (New England Nuclear Corp.) for 4 hr at 37°. Lysis of cells was performed in 1 ml of Tris-buffered saline (pH 7.6) containing 1% Nonidet P-40, 1 m*M* dithiothreitol, 2 m*M* phenylmethylsulfonyl fluoride, and 2 m*M* *N*-tosylphenylalanine chloromethyl ketone. The labeled immunoreactive proteins were then immunoprecipitated with antibovine insuline serum. For competitive binding studies, the antiserum was first neutralized with bovine insulin (2 $\mu$g of bovine insulin per 6 $\mu$l of antiserum for 30 min at 4°), after which the mixture was added to the samples. Examples of the immunoprecipitated material from cell extracts (Fig. 5A) and from the medium of the transformed cells (Fig. 5B) are shown.

## Comments

There appear to be two limitations in using BPV as eukaryotic cloning vector: (*a*) the limited host range due to the dependence of the BPV-induced transformed phenotype as the selective marker; and (*b*) the cis-inhibition by pBR322 sequence on BPV-induced transformation. These two shortcomings, however, may now be circumvented.

The host range of this BPV vector system may be extended by the use of a dominant selective marker in conjunction with the BPV transforming region. Experiments have been performed to evaluate the utility of the *E. coli* xanthine-quanine phosphoribosyltransferase (XGPRT) gene (*gpt*) as the dominant selective marker for the BPV-induced transformation of C127 cells.[14] A 2.2 kb *Bam*HI fragment containing the *E. coli gpt* harbored in a modified transcriptional unit of the SV40 early region[25] was inserted into the unique *Bam*HI of pBPV$_{69T}$ (52-1), and the resulting recombinant molecules, after the removal of pBR322 sequences were transfected onto C127 cells. Transformants were selected for the expression of the BPV transforming region, which induced phenotypically transformed foci in regular medium, or for the expression of the *E. coli gpt*, which enabled transformed colonies to grow in HAT medium containing xanthine and mycophenolic acid.[14] Cell lines established from individual foci selected for one phenotype were then assayed for the second phenotype. A tight linkage between the two selective markers was established in that 30 of 36 lines transformed with the recombinant DNA molecules

[25] R. C. Mulligan and P. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 2072 (1981).

TABLE II
TRANSFORMATION OF MOUSE CELLS BY BPV HYBRID PLASMIDS

| Plasmid | Bacterial segment | BPV DNA insert | Transformants/$\mu$g/$10^6$ cells | |
|---|---|---|---|---|
| | | | Cleaved[a] | Uncleaved |
| $pBPV_{69T}$ (17-6) | pBR322 | 69% transforming segment[b] | 81, 57 | 2, 5 |
| $pBPV_{69T}$ (54-2) | pML2 | 69% transforming segment[b] | 56, 46 | 1, 3 |
| pBPV (8-2) | pBR322 | *Bam*HI linear | >200 | 2, 5 |
| pBPV (142-6) | pML2 | *Bam*HI linear | >200 | >200 |

[a] The hybrid recombinant DNA has been cleaved at the restriction endonuclease sites forming the BPV DNA/prokaryotic plasmid junctions.
[b] The subgenomic transforming segment is the large *Hin*dIII–*Bam*HI fragment.

exhibited co-expression of the two phenotypic traits. The orientation of the *E. coli gpt* insert relative to the direction of BPV-1 transcription had no effect on expression. When DNA of these cell lines were examined by blot hybridization, the majority of these lines contained the hybrid DNAs in a plasmid state. It should be noted that a high proportion of the cell lines selected exhibited DNA rearrangement in these molecules. The reasons for these rearrangements are not known. This instability could be a result of the expression of the *E. coli gpt* in eukaryotic cells; alternatively, it could be the result of specific SV40 DNA sequences present in the $BPV_{69T}$-SV2gpt hybrid molecules, or finally, this instability may be inherent to the use of the 69% subgenomic transforming segment as a vector (see below). Experiments are underway utilizing other dominant selective markers in conjunction with other transcriptional regulatory signals.

The cis-inhibition of pBR322 on BPV DNA-mediated transformation has limited the effective use of BPV DNA as a plasmid vector for shuttling genes between eukaryotic cells and bacteria. More recent results, however, demonstrate that this limitation can be circumvented. It appears that the sequences in pBR322 that are inhibitory to the replication of SV40 sequences in monkey cells[13] may also be responsible for the inhibition of BPV plasmid replication in mouse cells, and hence BPV DNA-mediated transformation of mouse cells. Whereas the complete BPV-1 genome cloned in pBR322 at the *Bam*HI site can efficiently transform C127 cells only after cleavage from the pBR322 sequences, the same full-length BPV-1 DNA cloned into pML2, the "poison sequence" minus derivative of pBR322 can efficiently transform mouse cells with or without such cleavage (Table II).[26] Surprisingly, the 69% subgenomic transforming seg-

[26] N. Sarver, J. C. Byrne, and P. M. Howley, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7147 (1982).

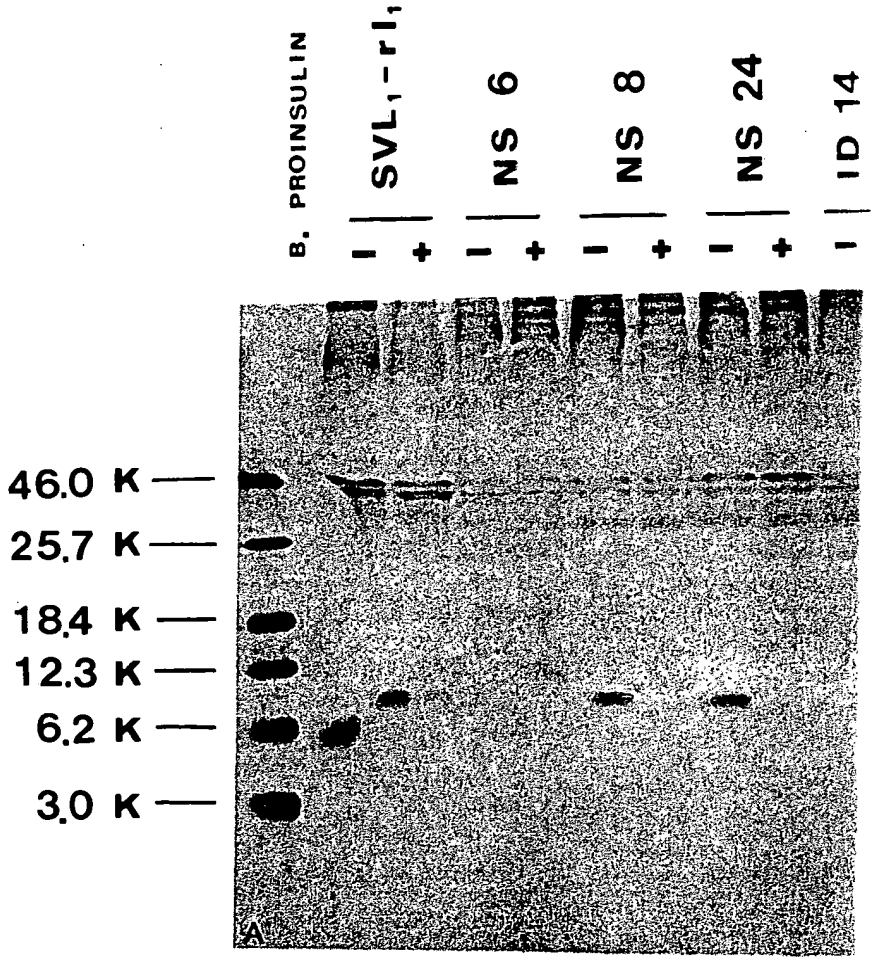


FIG. 5. Cells in 100-mm plates were labeled with 715 $\mu$Ci (200 $\mu$Ci/ml) of [$^{35}$S]cysteine for 4 hr at 37°, and the proteins were recovered as described.[2] Samples equivalent to medium from $10^6$ cells or extracts from $1.5 \times 10^6$ cells were immunoprecipitated with hamster antibovine serum for 16 hr at 4°. In competitive binding studies the antiserum was first neutralized with 2 $\mu$g of bovine insulin (30 min on ice) before it was added to the samples. Immunoprecipitated proteins were analyzed on sodium dodecyl sulfate–polyacrylamide gels. NS6, NS8, and NS24 are cells transformed by $BPV_{69T}$-$rI_1$ DNA. ID14 are cells transformed by bovine papillomavirus. (−) No competition; (+) with competition. (A) Analysis of cellular extracts on a 10 to 17% polyacrylamide linear gradient gel. (B) Analysis of proteins secreted into the medium on a linear 16% polyacrylamide gel. Migration of $^{14}$C-labeled markers and $^{14}$C-labeled bovine proinsulin is indicated. Reprinted, with permission, from Sarver *et al.*[2]

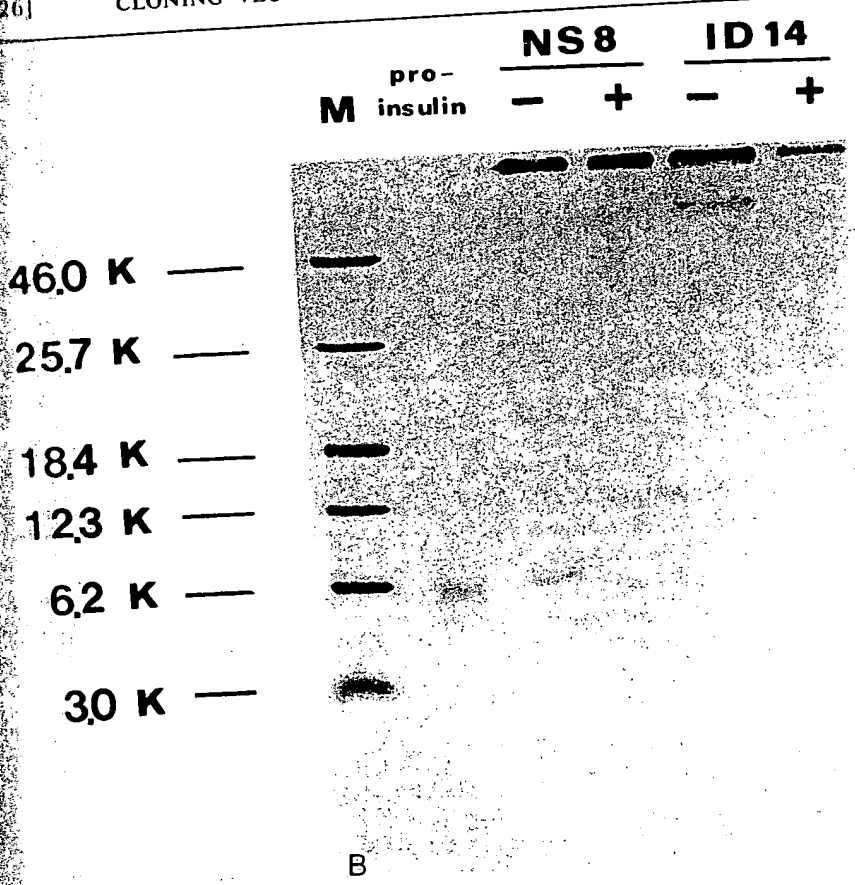


FIG. 5. (*Continued*)

ment cloned into pML-2 will transform mouse cells only after cleavage from the prokaryotic sequences. This suggests that some regulatory region may be present in remaining 31% of the BPV-1 genome that can affect the expression of the transforming segment of BPV-1. Interestingly, a number of eukaryotic gene segments have been shown to have a similar effect as this 31% BPV DNA segment when cloned into a $BPV_{69T}$–pML2 recombinant. A 7.6 kb segment of the human $\beta$-globin gene inserted into a $BPV_{69T}$–pML2 hybrid can be used to transform mouse cells efficiently.[27] The BPV–pML2–$\beta$-globin hybrids are maintained in the transformed cells as plasmids and can be recovered from the transformed cells

[27] D. DiMaio, R. Treisman, and T. Maniatis, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4030 (1982).

by transformation of susceptible bacteria, thus demonstrating the ability to use BPV–pML2 hybrids as shuttle vectors.[27] In our laboratory we have demonstrated a similar activity in a human growth hormone DNA segment, a 5.3 kb rat preproinsulin DNA segment, and a rat α-fibrinogen DNA segment.[28] Each of these DNA segments when cloned into a $BPV_{69T}$–pML2 hybrid stimulate the transformation efficiency of mouse C127 cells. The nature of the sequences that result in this stimulation and the molecular basis for this biological effect are not known and are under investigation.

## Acknowledgments

We are grateful to Ms. S. Hostler for excellent editorial assistance in the preparation of this manuscript.

[28] N. Sarver, S. Mitrani-Rosenbaum, M.-F. Law, J. C. Byrne, and P. M. Howley, *in* "Gene Transfer and Cancer" (N. Steinberg and M. Pearson, eds.), in press. Raven, New York, 1982.

# [27] High-Efficiency Transfer of DNA into Eukaryotic Cells by Protoplast Fusion

*By* ROZANNE M. SANDRI-GOLDIN, ALAN L. GOLDIN, MYRON LEVINE, and JOSEPH GLORIOSO

Protoplast fusion is a method for directly transferring cloned DNA from bacteria to mammalian cells at high frequency. This technique, unlike calcium phosphate precipitation,[1–5] DEAE-dextran precipitation,[6] direct microinjection,[8–11] or the use of liposomes as carrier vehicles,[12–14]

[1] F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973).
[2] N. J. Maitland and J. K. McDougall, *Cell* **11**, 233 (1977).
[3] M. Wigler, S. Silverstein, L.-S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel, *Cell* **11**, 223 (1977).
[4] M. Wigler, A. Pellicer, S. Silverstein, and R. Axel, *Cell* **14**, 725 (1978).
[5] K. M. Huttner, G. A. Scangos, and F. H. Ruddle, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5820 (1979).
[6] J. H. McCutchan and J. S. Pagano, *J. Natl. Cancer Inst.* **41**, 351 (1968).
[7] G. Milman and M. Herzberg, *Somatic Cell Genet.* **7**, 161 (1981).
[8] E. G. Diacumakos, *Methods Cell Biol.* **7**, 287 (1973).
[9] C.-P. Lui, D. L. Slate, R. Gravel, and F. H. Ruddle, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4503 (1979).
[10] M. R. Capecchi, *Cell* **22**, 479 (1980).




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does not require the isolation or purification of the cloned DNA sequences to be transferred. As first reported by Schaffner,[15] cloned SV40 DNA was transferred directly from bacteria to mammalian cells by converting the bacteria to protoplasts and then fusing with mammalian cells using polyethylene glycol. We have modified the protoplast fusion procedure of Schaffner[15] and have shown that cloned herpes simplex virus type 1 (HSV-1) sequences can be transferred to mammalian cells and expressed at high frequency.[16] Furthermore, stable transformation of Ltk$^-$ cells[17] to a tk$^+$ phenotype by the HSV-1 *tk* (thymidine kinase) gene also occurred at high frequency in these studies.[16] Here we describe our procedure for protoplast fusion (see Fig. 1).

## Principle of the Method

The direct transfer of cloned DNA sequences from bacteria to eukaryotic cells involves only two steps: the conversion of the bacteria to protoplasts or spheroplasts by digestion of the cell wall with lysozyme, and the fusion of the bacterial protoplasts to the animal cells with polyethylene glycol. The second step presumably results in membrane fusion and hybrid cell formation, although this has not been demonstrated in the case of bacterial protoplasts and animal cells. In fact, ultrastructural analysis of plant cell protoplasts fused to bacterial spheroplasts indicated that the spheroplasts were taken into the plant cell cytoplasm by endocytosis, fusion being observed only at low frequency.[18] Whether the transfer occurs by membrane fusion or by endocytosis, genetic material carried by the bacteria is released into the eukaryotic cells and expressed at high frequency.[15,16,18–20]

[11] A. Graessmann, H. Wolf, and G. W. Bornkamm, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 433 (1980).
[12] R. Fraley, S. Subramani, P. Berg, and D. Papahadjopoulos, *J. Biol. Chem.* **255,** 10431 (1980).
[13] T.-K. Wong, C. Nicolau, and P. H. Hofschneider, *Gene* **10,** 87 (1980).
[14] M. Schaefer-Ridder, Y. Wang, and P. H. Hofschneider, *Science* **215,** 166 (1982).
[15] W. Schaffner, *Proc. Natl. Acad. Sci. U.S.A.* **77,** 2163 (1980).
[16] R. M. Sandri-Goldin, A. L. Goldin, M. Levine, and J. C. Glorioso, *Mol. Cell. Biol.* **1,** 743 (1981).
[17] S. Kit, D. R. Dubbs, L. J. Piekarski, and T. C. Hsu, *Exp. Cell. Res.* **31,** 297 (1963).
[18] S. Hasezawa, T. Nagata, and K. Syono, *Mol. Gen. Genet.* **182,** 206 (1981).
[19] B. R. de Saint Vincent, S. Delbrück, W. Eckhart, J. Meinkoth, L. Vitto, and G. Wahl, *Cell* **27,** 267 (1981).
[20] M. Rassoulzadegan, B. Binetruy, and F. Cuzin, *Nature* (*London*) **295,** 257 (1982).

## Materials and Methods

### *Eukaryotic Cell Lines*

High-frequency transfer and expression of genetic sequences following protoplast fusion has been demonstrated with a variety of cell lines. We have used mouse Ltk$^-$ cells and Vero cells (an African green monkey kidney cell line).[16] In addition, two other African green monkey kidney cell lines, CV-1[15,20] and BSC-1,[15] have been used as well as HeLa cells (a human cervix carcinoma line),[15] WI-38 (a diploid human fibroblast line),[21] FR3T3 (a rat cell line)[20] 3T6 (a mouse fibroblast line),[15] and two Chinese hamster ovary cell lines (CHO-K1 and Urd$^-$A).[19] In addition to mammalian cell lines, plant cells (*Vinca rosea*) have been used as recipients in fusion experiments.[18]

### *Bacterial Strains*

The *Escherichia coli* strains that have been used include DH-1,[16] HB101,[15,19] and 1106.[20] *Agrobacterium tumefaciens* was used in fusions with plant cells.[18] The choice of bacterial strain may be an important consideration. While Schaffner[15] and de Saint Vincent *et al.*[19] have reported successful transfer using HB101, we found that this strain required a longer lysozyme treatment than DH-1 for conversion to protoplasts and once protoplasts were formed they lysed more readily than DH-1 protoplasts.[21] Rassoulzadegan *et al.*[20] found strain 1106 to be quite efficient in producing stable protoplasts.

### *Chimeric Plasmids*

Cloned DNA sequences shown to be expressed following transfer by protoplast fusion include the HSV-1 *tk* gene,[16] HSV-1 sequences within an *Eco*RI fragment from the long region of the HSV-1 genome,[16] SV40 viral DNA,[15,20] polyoma early genes,[20] a CAD gene from Syrian hamster cells,[19] an *E. coli* GPT (guanine–xanthine phosphoribosyltransferase) gene linked to eukaryotic control signals,[19] and an octopine-type Ti plasmid.[18]

### *Formation of Protoplasts*

*Escherichia coli* K12 strain 1100 derivative DH-1 (*rec A1 hsd R hsd M$^+$ Nal A96$^R$ thi-1 end A1 supE 44*)[16] was used as the donor for our protoplast fusion experiments. DH-1 bacteria carrying the appropriate plas-

[21] R. Sandri-Goldin and A. L. Goldin, unpublished observations, 1982.

mids were grown at 37° in 50 ml of M9 salts[22] containing 0.5% casamino acids, 0.4% glucose, 0.012% $MgSO_4$, 5 μg of thiamine per milliliter, and 50 μg of ampicillin per milliliter to an absorbance at 600 nm of 0.7 to 0.8 (about $5 \times 10^8$ cells per milliliter). This generally took 5.5–6 hr. Chloramphenicol or spectinomycin (when the plasmid carried a chloramphenicol-resistance gene) was added to 250 μg/ml, and the culture was incubated at 37° for an additional 12–16 hr to amplify the plasmid copy number.[23] We and others[20] have found the chloramphenicol amplification step to be essential for successful transformation following protoplast fusion. No $tk^+$ transformants were observed in experiments in which the amplification was omitted.[21] Similarly, Rassoulzadegan *et al.*[20] found no polyoma transformants when this step was eliminated. In contrast, Schaffner[15] observed only a twofold enhancement in the percentage of cells expressing SV40 T antigen when amplification was used. This difference in results may be due to the assays used to measure expression. In our experiments and those of Rassoulzadegan *et al.*[20] transformant colonies were scored, whereas Schaffner[15] observed antigen expression shortly after fusion. It is possible that more copies of chimeric plasmid DNA must be introduced into the cell to ensure that stable transformation occurs while even a small number of copies might result in transient gene expression.

After incubation with chloramphenicol, the bacteria were transferred to 25-ml Corex centrifuge tubes, centrifuged at 3000 *g* for 10 min at 4°, and suspended in a total volume of 2.5 ml of chilled 20% sucrose in 0.05 *M* Tris-HCl (pH 8.0). Lysozyme (Millipore Corporation, Freehold, New Jersey) was added (0.5 ml of a freshly prepared solution of 5 mg of lysozyme per milliliter in 0.25 *M* Tris-HCl, pH 8.0), and the mixture was held on ice for 5 min. One milliliter of 0.25 *M* EDTA (pH 8.0) was added to the suspension, which was held on ice for an additional 5 min, after which 1.0 ml of 0.05 *M* Tris-HCl (pH 8.0) was added. The suspension was incubated at 37° until at least 90% of the bacteria were converted to protoplasts as monitored by phase-contrast microscopy. For strain DH-1 this required about 10 min, although careful monitoring was necessary. Low transfer frequencies resulted from either too long an incubation at 37°, causing substantial lysis of the protoplasts, or too short an incubation with lysozyme, so that too few of the bacteria were converted to protoplasts. The protoplast suspension was then slowly and carefully diluted with 20 ml of prewarmed MEM (Eagle minimal essential medium supplemented with

[22] J. H. Miller, "Experiments in Molecular Genetics," p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1972.

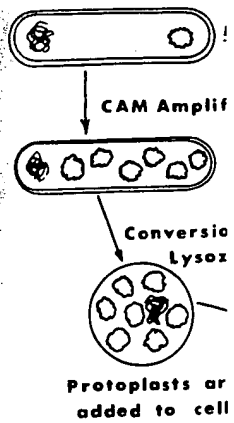
[23] V. Hershfield, H. W. Boyer, C. Yanofsky, M. A. Lovett, and D. R. Helinski, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3455 (1974).

nonessential amino acids, 100 μg of streptomycin per ml and 100 U of penicillin per milliliter) containing 10% sucrose and 10 m*M* $MgCl_2$. Lysis of the protoplasts occurred if the MEM was added too quickly. MEM was best added dropwise while gently agitating the tube to mix the solution. After the addition of 7.5–10 ml of medium in this manner, the remainder was added somewhat more quickly. At this point the suspension was kept at ambient temperature for the remaining steps. The final suspension contained about $10^9$ bacteria per milliliter and was added directly to the cell monolayers.

*Fusion of Protoplasts to Cell Monolayers (see Fig. 1)*

Tissue culture cells were seeded in 24-well or 6-well dishes at 12–24 hr before the fusion so that cell monolayers were subconfluent (about 2 to $4 \times 10^4$ cells/well of a 24-well dish and about $10^5$ cells/well of a 6-well dish). Subconfluent monolayers were used to minimize cell-to-cell fusion, which results in polykaryocyte formation and reduced survival of fused cells. Medium was removed from the cells and either 1.0 ml (24-well dish) or 2.0–4.0 ml (6-well dish) of protoplast suspension was added to each well. The ratio of protoplasts to cells was about 2 to $4 \times 10^4$:1. The protoplasts were pelleted onto the cells by centrifuging at 3000 rpm for 15 min in a swinging Microtiter dish TH-4 rotor of a Beckman TJ-6 centrifuge. As this rotor was designed for Microtiter dishes, not 24-well or 6-well dishes, the latter may be difficult to fit into the rotor. The dishes we used are made by Costar and fit snugly into the rotor, although cracking of the top of the dish occurs occasionally. Spare sterile dishes are kept nearby to replace tops as necessary. After centrifugation, the supernatant was removed by aspiration and the dish was drained by gently tilting. Care must be taken to prevent sloughing the pelleted protoplasts from the cell monolayer. Two milliliters of polyethylene glycol solution (PEG) was added to each well of a 6-well dish or 1.0 ml was added to each well of a 24-well dish. The PEG solution consisted of 50 g of PEG-1000 (Sigma Chemical Co.) in 50 ml of MEM. This solution is most easily made by melting the PEG-1000 at 65°, pouring 50 g into a 100-ml bottle, autoclaving the PEG in the bottle, and then adding 50 ml of MEM after the molten PEG has cooled somewhat but has not yet solidified (about 65°). The PEG solution was left on the cells for different times in various experiments. In general, a 2-minute fusion time has worked well with $Ltk^-$ cells as well as Vero cells, although Vero cells survive well after fusions of up to 4 min. The fusion time may have to be adjusted depending on the cell line used. Some cell lines, such as the HeLa derivative BU-25 or HEF, survived poorly after even short fusion times, so that the PEG concentration may have to be lowered with these cells. After the chosen time, the PEG solution was



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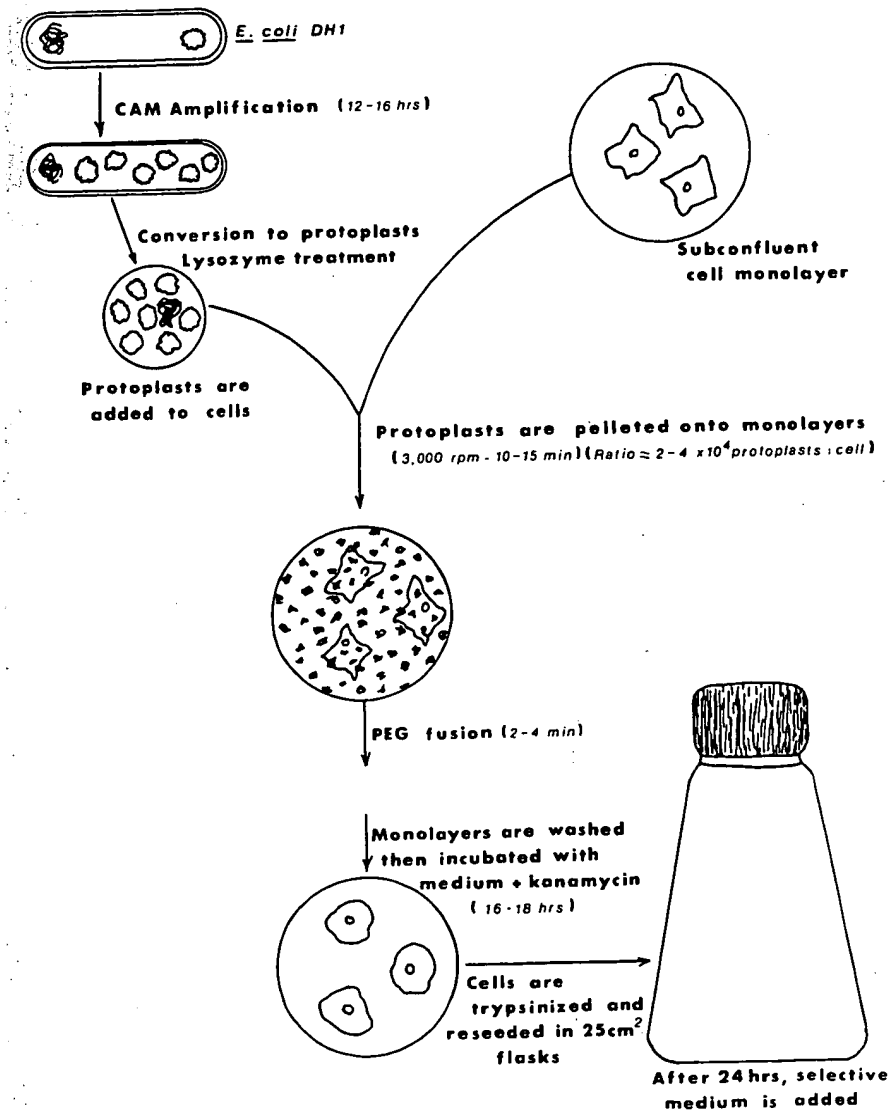


FIG. 1. Schematic representation of the procedure for DNA transfer by protoplast fusion.

removed and the plates were rinsed rapidly with 4 ml of MEM (1.5 ml for 24-well dishes) three successive times, and fresh MEM containing 10% fetal calf serum and 100 $\mu$g/ml kanamycin was added to each well. The kanamycin was included to prevent the growth of any bacteria which escaped conversion to protoplasts. Fused monolayers were incubated at 37°.

Fused cells to be analyzed by immunofluorescence were seeded initially onto glass coverslips at the bottom of the wells of 24-well dishes. At various times after fusion (usually 12–48 hr), the cells were fixed in acetone and then treated with the appropriate antibodies. When $tk^+$ transformant colonies were to be isolated, the fused cells were incubated at 37° for about 16–18 hr after fusion, at which time the medium was removed and fresh MEM containing 10% fetal calf serum was added. Incubation at 37° was continued for an additional 8–12 hr, at which time cells were trypsinized, serially diluted, and reseeded in 25 $cm^2$ flasks. After 24 hr, medium containing HAT (15 $\mu$g of hypoxanthine, 1 $\mu$g of aminopterin, 5 $\mu$g of thymidine, and 15 $\mu$g of glycine per milliliter[3]) was added to the flasks to select for $tk^+$ transformants. Medium without HAT was added to additional flasks to determine the total number of cells surviving the fusion procedure. Cells were refed every 3 days. Unselected colonies were counted after 1 week, and $tk^+$ colonies were scored after 2 weeks. The colonies were picked with sterile toothpicks into 24-well dishes after 3 weeks and were routinely grown in MEM with 10% fetal calf serum containing HAT.

## Results and Discussion

The transfer of material from protoplasts to animal cells was initially monitored by labeling DH-1 protoplasts with the fluorescent dye fluorescein isothiocyanate (FITC).[16] One milligram of FITC was added to the protoplast suspension after the addition of 0.05 *M* Tris-HCl. The labeled protoplasts were fused to Vero cells grown on coverslips for 1, 2, 3, 4, or 5 min. One hour after the fusion, cells were fixed in acetone and observed under a microscope with epifluorescent illumination. In early experiments, around 1–5% of the cells fluoresced after 4- or 5-min fusions, whereas in some later experiments up to 50% of the cells fluoresced after 2- to 4-min fusions. Fluorescence was not observed when protoplasts were pelleted onto monolayers, but no PEG treatment was used.

That DNA was transferred to the cells as a consequence of protoplast fusion was shown by *in situ* hybridization.[16] Protoplasts containing the plasmid pBR325[24] were fused to Vero cells (grown on coverslips) for 90 sec. Twenty-one hours after the fusion, cells were fixed, the DNA was denatured by boiling in 0.1 × SSC, and $^{32}$P-labeled pBR325 DNA was hybridized to the cells. In this experiment it was estimated that about 1–2% of the cells showed hybridization to plasmid DNA.

To determine whether transferred sequences were expressed, bacteria

[24] F. Bolivar, *Gene* **4**, 121 (1978).

carrying the plasmid pSG18[25] containing a 16.5 kb HSV-1 *Eco*RI fragment inserted into pBR325 were fused to Vero cells, which were subsequently analyzed by immunofluorescence with antibody made against purified HSV-1 virions.[16] About 5% of the cells fused with these protoplasts fluoresced, whereas no fluorescence was observed when the fusion was done with protoplasts carrying pBR325 but no HSV-1 seqences. This level of expression was consistent with the level of SV40 T antigen expression observed by Schaffner,[15] who fused CV-1 cells with strain HB101 protoplasts carrying the plasmid pBSV-3X which contains three tandem copies of the SV40 genome. More recently, Rassoulzadegan *et al.*[20] reported that as many as 100% of CV-1 cells and around 50% of FR3T3 cells were positive for T antigen expression when these cells were fused with strain 1106 bacteria carrying the plasmid pSV-1, which contains the SV40 early region. This high percentage of transfer was observed at bacteria : recipient cell ratios of about $10^4$. Transfer was less efficient at lower ratios. As these authors have pointed out, differences in expression may reflect differences either in the efficiency of fusion or the level of expression of various genes, for example HSV-1 genes as compared to SV40 early genes.

In addition to expression, transformation was found at high frequency following gene transfer by protoplast fusion. We transferred the plasmid pX1,[26] which contains the HSV-1 *tk* gene in pBR322 to Ltk$^-$ cells.[16] Tk$^+$ colonies (assayed by counting colonies after 2 weeks in HAT selective medium) were found at frequencies of up to one cell in 300–500, though frequencies of about 1 in $10^3$ are more usual. No tk$^+$ colonies were found when pBR322 alone was transferred. High frequencies of stable transformation have also been observed by others. Rassoulzadegan *et al.*[20] found the efficiency of focus formation following transfer of either polyoma or SV40 early genes in pBR322 plasmids was about 40 transformants per $2 \times 10^5$ cells at an input ratio of $10^4$ protoplasts per cell. These values are comparable to those observed after infection of the same cells with high multiplicities of viral particles.[20,27] Transformation frequencies as high as 0.1 to 0.5% of the recipient cells were also observed by de Saint Vincent *et al.*,[19] who transferred three chimeric plasmids: the Syrian hamster CAD gene cloned in a cosmid vector, the cloned *E. coli GPT* gene,[28,29] and a functional cDNA clone of the mouse dihydrofolate reductase gene.[30]

[25] A. L. Goldin, R. M. Sandri-Goldin, M. Levine, and J. C. Glorioso, *J. Virol.* **38,** 50 (1981).
[26] L. W. Enquist, G. F. Vande Woude, M. Wagner, J. R. Smiley, and W. C. Summers, *Gene* **7,** 335 (1979).
[27] R. Seif and F. Cuzin, *J. Virol.* **24,** 721 (1977).
[28] R. Mulligan and P. Berg, *Science* **209,** 1422 (1980).
[29] R. Mulligan and P. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2072 (1981).
[30] S. Subramani, R. C. Mulligan, and P. Berg, *Mol. Cell. Biol.* **1,** 854 (1981).

The transformed cell lines resulting from protoplast fusion gene transfer were stable in nonselective medium. We found that 7 cell lines grown from individual $tk^+$ colonies plated with equal efficiency in selective and nonselective media as soon as they could be tested after transformation and after 5 weeks in nonselective medium.[21] de Saint Vincent *et al.*[19] investigated the stability of five different CAD transformants and found equal plating efficiencies in selective and nonselective media early and after propagation of the lines in nonselective medium for over 100 generations. This stability is comparable to that seen in the stable transformants resulting from microinjection[10] or transfection by calcium phosphate precipitation,[3,31] although numerous instances of unstable transformants have been seen with transfection.[3,31,32] While it has not been reported with transfection or microinjection, we found that protoplast fusion resulted in about 20—30% abortively transformed colonies, which generally did not grow beyond 50–60 cells in selective medium.[21]

The stability of the successfully transformed cell lines may be a direct result of the integration of the transforming DNA into the chromosomal DNA of the recipient cell. Using Southern transfer analysis,[33] we have found that transforming tk DNA was integrated into high molecular weight DNA,[34] and de Saint Vincent *et al.*[19] reported that CAD gene DNA was similarly integrated into high molecular weight DNA. de Saint Vincent *et al.*[19] also used *in situ* hybridization to chromosomal metaphase spreads to demonstrate that most, possibly all, of the CAD gene DNA was associated with a single chromosomal region in each transformed cell line. This suggests integration into cellular genomic DNA.

We have analyzed the structure of the integrated DNA in three $tk^+$ (pX1) transformed cell lines by Southern transfers using four restriction endonucleases that cleave the pX1 plasmid in 0 to 3 sites, respectively. The resulting filters were hybridized to the purified HSV-1 tk transforming fragment of pX1, to the pBR322 vector fragment of pX1, and to *E. coli* DH-1 bacterial DNA. The following preliminary results were obtained.

1. The pX1 plasmid DNA was integrated primarily (if not exclusively) in one site in each of the recipient cell lines.
2. The entire plasmid, including the pBR322 sequences, was present in each cell line.
3. No rearrangements of the plasmid DNA were detectable.

[31] G. A. Scangos, K. M. Huttner, D. J. Juricek, and F. H. Ruddle, *Mol. Cell. Biol.* **1**, 111 (1981).
[32] L. H. Graf, G. Urlaub, and L. Chasin, *Somatic Cell Genet.* **5**, 1031 (1979).
[33] E. M. Southern, *J. Mol. Biol.* **98**, 503 (1975).
[34] R. M. Sandri-Goldin, A. L. Goldin, M. Levine, and J. C. Glorioso, in preparation.

4. The plasmid was integrated in a multimeric configuration in all of the cell lines.

No hybridization of *E. coli* genomic DNA was found with any of the pX1 fragments in the transformed cell lines. This does not rule out the presence of bacterial genomic DNA in the transformants, however, because the highly complex bacterial DNA is an insensitive hybridization probe. All the data are consistent with the integration of an intact multimeric plasmid into a single chromosomal site resulting in transformation of the recipient cell in these three cell lines.[34]

## Summary

Protoplast fusion is a highly efficient method for effecting gene transfer to cells in culture resulting in stable transformation at high frequency. A number of cell lines have been used successfully as recipients. There is no need to isolate and purify the DNA, which not only saves time and effort but eliminates steps that cause nicking or breaking of large cloned inserts. The high-frequency transformation achievable by protoplast fusion should make this procedure useful for studies on gene expression and for screening cloned genomic libraries for genes that can be expressed in recipient cells.

## Acknowledgments

This work was supported by Grants 5P40-RR00200, AI18228, and AI17900 from the National Institutes of Health.

# [28] Gene Transfer into Mouse Embryos: Production of Transgenic Mice by Pronuclear Injection

*By* JON W. GORDON and FRANK H. RUDDLE

Transgenic mice are mice into which have been transferred cloned genetic material. Techniques for production of such mice have only recently been developed.[1] Consequently, exploitation of transgenic mice for studies of mammalian gene regulation has only just begun. These studies have thus far shown that transferred sequences can be retained throughout embryonic development until birth,[1] that they can become integrated into the host genome and transmitted through the germ line to succeeding genera-

[1] J. W. Gordon, G. A. Scangos, D. J. Plotkin, J. A. Barbosa, and F. H. Ruddle, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7380 (1980).




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